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Authorized Signature:

Mary C. Gawlicki
President
Corporate Translations, Inc.

January 6, 2000

"Subscribed and sworn to before me this 6th day of January, 2000"

Notary Public
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18A LEDGEBROOK DRIVE • MANSFIELD CENTER, CT 06250 USA • TEL: 860-450-0405 • FAX: 860-450-0409

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Paris, December 10, 1999

For the Director General of the
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Martine PLANCHE

NATIONAL INSTITUTE OF INDUSTRIAL PROPERTY

MAIN OFFICE
26 bis, rue de Saint Petersbourg
75800 PARIS Cedex 08
Telephone: 01 53 04 53 04
Fax: 01 42 93 59 30

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REQUEST FOR DELIVERY OF A CERTIFICATE OF INDUSTRIAL PROPERTY*

1

a X PATENT OF INVENTION
b CERTIFICATE OF UTILITY
c DIVISIONAL APPLICATION
d CONVERSION OF A EUROPEAN PATENT OF INVENTION

For c and d, specify: Type, No. and date of initial application

2 MANDATORY OPTIONS when filing (except for certificate of utility)

THE APPLICANT REQUESTS **POSTPONEMENT** OF THE SEARCH REPORT — YES X NO

IF THE OPTION SELECTED IS NO AND IF THE APPLICANT IS AN INDIVIDUAL, HE REQUESTS STAGGERED PAYMENT OF THE SEARCH REPORT FEE — YES X NO

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7 TITLE OF THE INVENTION

MEDICINAL ASSOCIATION USEFUL FOR THE *IN VIVO* TRANSFECTION AND EXPRESSION OF EXOGENES

8 APPLICANT(S): First and last names (underline last name) or name and legal form

SIREN No.

CNRS: NATIONAL CENTER FOR SCIENTIFIC RESEARCH
IGR: INSTITUT GUSTAVE ROUSSY
INSERM: NATIONAL INSTITUTE OF HEALTH AND MEDICAL RESEARCH

9 COMPLETE ADDRESS(ES)	COUNTRY
3, Rue Michel-Ange – 75016 PARIS 39, Rue Camille Desmoulins – 94800 VILLEJUIF 101, Rue de Tolbiac – 75013 PARIS CEDEX 13	FRANCE

10 NATIONALITY(IES)
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11 INVENTOR(S)
THE APPLICANT IS THE SOLE INVENTOR — YES / NO
If the answer is no, see explanatory note

12 IF THE APPLICANT IS A NON-ASSESSABLE INDIVIDUAL, HE REQUESTS* OR HAS REQUESTED REDUCTION OF THE FEES — YES / NO

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15 SIGNATURE OF APPLICANT OR AGENT AND NAME AND POSITION OF SIGNATORY – REGISTRATION No. [signature] Pascale LE COUPANEC	SIGNATURE OF RECEPTION ATTENDANT	SIGNATURE AFTER REGISTRATION OF APPLICATION AT THE NIIP [signature]

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26 bis, rue de Saint-Pétersbourg 75800 Paris Cédex 08 Telephone: (1) 42 94 52 52 – Fax: (1) 42 93 59 30

Patent Administration Division

NAME OF THE INVENTOR (if the applicant is not the inventor or sole inventor	National Registration No. 9501662

Title of the Invention:

MEDICINAL ASSOCIATION USEFUL FOR THE *IN VIVO* TRANSFECTION AND EXPRESSION OF EXOGENES

The undersigned NATIONAL CENTER FOR SCIENTIFIC RESEARCH, 3 Rue Michel-Ange, 75016 PARIS – INSTITUT GUSTAVE ROUSSY, 39, Rue Camille Desmoulins 94800 VILLEJUIF – FRANCE – NATIONAL INSTITUTE OF HEALTH AND MEDICAL RESEARCH , 101, Rue Tolbiac, 75013 PARIS CEDEX 13

designate as the inventor(s) *(indicate first and last names and address and underline last name):*

CHATENOUD Lucienne: 14, Rue des Pavillons, 92800 PUTEAUX – FRANCE

HADDADA Hedi: 1, Rue Jules Guesde, Appt 221, 94140 ALFORTVILLE – FRANCE

LEE Martin: 9, Rue Sampaix, 75010 PARIS – FRANCE

PERRICAUDET Michel: 31, Rue de Chartres – 28320 ECROSNES – FRANCE

NOTE: In exceptional cases, the name of the inventor can be followed by that of the company to which he belongs (company that employs him) when the latter is different from the applicant or proprietary company.

Date and signature(s) of the applicant(s) or agent

Antony, February 14, 1995
[signature]

Pascale LE COUPANEC

BA 113/160392

DOCUMENT INCLUDING CHANGES

PAGE(S) OF THE DESCRIPTION OR CLAIMS OR PLATE(S) OF DRAWING			MC*	DATE OF CORRESPONDENCE	DATE STAMP OF EXAMINER
Modified	Deleted	Added			
24				25 APRIL 1995	[illegible] 04 MAY 1995 [poorly legible]

Any change in the wording of the original claims, unless it follows from the provisions of Article 28 of the Decree of September 19, 1979, is indicated by the notation "MC" (modified claims).

The present invention pertains to the field of gene therapy and notably to the use of adenoviruses for the expression of genes of therapeutic interest. More particularly, it pertains to a new method of treating diseases of genetic origin based on the combined use of two types of therapeutic agents.

Gene therapy consists of correcting a deficiency or an anomaly (mutation, aberrant expression, etc.) by introducing genetic information into the affected cell or organ. This genetic information can be introduced either *in vitro* or *ex vivo* into a cell extracted from the organ, with the modified cell then being reintroduced into the organism, or directly *in vivo* into the appropriate tissue. In the second case, there are different physical transfection techniques, including the use of viruses as vectors. In that regard, different viruses have been tested for their capacity to infect certain cell populations. In particular, retroviruses (RSV [respiratory syncytial virus], HMS, MMS, etc.), the HSV [herpes simplex virus] viruses, adeno-associated viruses and adenoviruses.

Among these viruses, the adenoviruses have certain properties that are advantageous for use in gene therapy. They have a fairly broad host spectrum, they are capable of infecting quiescent cells and they do not combine with the genome of the infected cell. Adenoviruses are viruses with linear double strand DNA of approximately 36 kb. Notably, their genome comprises a repetitive inverse sequence (ITR) at the end, an encapsidation sequence, early genes and late genes (cf Figure 1). The principal early genes are the E1 (E1a and E1b), E2, E3 and E4 genes. The principal late genes are the L1 to L5 genes.

Considering the properties of the aforementioned adenoviruses, the latter have already been used for gene transfer *in vivo*. In that regard, different vectors derived from adenoviruses have been prepared, incorporating different genes (ß-gal, OTC, α-1AT, cytokines, etc.). In each of these constructions, the adenovirus was modified to render it incapable of replication in the infected cell. Thus, the constructions described in the prior art are adenoviruses with E1 regions (E1a and/or E1b), and possibly E3 deleted, and into which a heterologous DNA sequence is inserted (Levrero et al., Gene 101 (1991) 195; Gosh-Choudhury et al., Gene 50 (1986) 161).

However, as for all known viruses, the administration of a wild-type (Routes et al., J. Virol. 65 (1991) 1450) or defective recombinant adenovirus for replication (Yang et al., PNAS (1994) 4407), induced an important immune response.

In fact, one of the major roles of the immune system is to destroy the non-self or self-altered elements in the body. To do this, the immune system develops two types of defensive action. The first consists in producing antibodies which will bind to the antigen in question in order either to inactivate it or to organize its removal from the body. In this case, we speak of humoral immunity. The second method of defense, which is called cellular immunity, makes use of cytotoxic T cells. These cells specifically attack the infected cells, that is, those that have acquired antigens on their surface. More precisely, the receptors of cytotoxic T cells recognize the antigens introduced in association with molecules of the class I major histocompatibility complex (MHC-I) on the surface of infected cells. This is followed by the destruction of the infected cell.

Consequently, this immune response developed against infected cells constitutes a major obstacle to the use of viral vectors in gene therapy since (i) by inducing the destruction of the infected cells, it limits the duration of expression of the therapeutic gene, and therefore, the therapeutic effect; (ii) at the same time, it induces an important inflammatory response, and (iii) it leads to the rapid elimination of the infected cells after repeated injections. It is understood that the amplitude of this immune response against infected cells varies according to the nature of the organ undergoing the injection and the injection method used. Thus, the expression of the ß-galactosidase, which is encoded by a recombinant adenovirus administered into the muscle of immunocompetent mice is reduced to minimum levels 40 days after the injection (Kass-Eisler et al., PNAS 90 (1993) 11498). Moreover, the expression of transfected genes by adenoviruses in the liver is significantly reduced within 10 days following the injection (Yang Y et al. 1994 Immunity 1 433-442), and the expression of factor IX which was transferred by the adenovirus into the hepatocytes of hemophilic dogs disappeared 100 days after the injection (Kay et al., PNAS 91 (1994) 2353).

From the standpoint of the use of vectors derived from adenoviruses in gene therapy, therefore, it seems to be necessary to control the immune response developed in opposition to them or against the cells that they infect.

From the preceding information, it is clear that activation of the immune system requires, first of all, recognition by the latter of the non-self or self-altered elements, such as for example, vectors derived from adenoviruses, which must be destroyed. A tolerance phenomenon arises from the recognition of self or non-self.

It is precisely at this level that the present invention intervenes. It is aimed at preventing the rapid elimination of adenoviruses from infected cells, and therefore, consequently prolonging the *in vivo* expression of the therapeutic gene that they deliver.

Recently, the Applicant has demonstrated that the co-expression of certain genes in the infected cells can induce an immunoprotective effect, thus, allowing the vectors and/or the infected cells to be released into the immune system. [The applicant] has notably developed adenoviruses in which the expression of a gene of therapeutic interest is coupled with that of an immunoprotective gene (FR No. 94 12346). Notably, this may be a gene whose product acts on the activity of the major histocompatibility complex (MHC) or on the activity of cytokines, thus permitting it to reduce considerably, or even to eliminate any immune reaction against the vector or the infected cells. They at least partially inhibit the expression of MHC proteins or the introduction of antigens with the advantageous result of a notable reduction in the immune reaction against the vector or the infected cells, and thus, a prolonged therapeutic effect.

The applicant has unexpectedly demonstrated that it was possible to prolong significantly over time the therapeutic effect of such a vector by associating it with an immunosuppressant. The destruction, by the immune system, of the vector considered or of the infected cells is delayed over time for a clearly longer period than that which could be expected from the simple juxtaposition of the immunoprotective effects induced by said vector and immunosuppressive agent, respectively. Advantageously, the drug association that is the subject of this invention induces a pseudo-inertia phenomenon of the immune system which is favorable to the prolonged expression over time of a therapeutic gene.

According to the invention, an immunosuppressive agent is any compound that is capable of partially or completely inhibiting at least one immune signaling pathway. In general, immunosuppressive agents are compounds that are classically administered following an organ transplantation in order to prevent any reaction rejecting the latter. The compounds that are classically used are either chemical agents, such as cyclosporin, FK506, azathioprine and corticosteroids, or monoclonal or polyclonal antibodies. With regard to the first category of immunosuppressive agents, their function is to prevent the synthesis of interluekin-2 and/or other lymphokines which play an important role in the growth and activity of lymphocytes. Unfortunately, the efficacy of this type of immunosuppressive agent requires continuous administration, which is known to have more or less long-term harmful toxic effects. Thus, azathioprine is a known bone marrow suppressant and cyclosporin is nephrotoxic and may also cause hypertension and neurological problems.

More particularly, with regard to the antibodies, they are antibodies directed against the lymphoid cells of the immune system. The primary antibody used as an immunosuppressive agent is anti-CD3, directed against T lymphocytes. Its target is one of the CD3 molecules that make up the T-cell receptor-antigen complex. The result is the inactivation of the immune cells carrying that molecule thus blocking activation of the immune system. Therefore, it would be incapable of reacting to the presence of infected cells, even though antigens are present. On the same principle, it is possible to make use of anti-CD4, -CD2, -CD8, -CD28, -B7, -ICAM-1 and –LFA1 antibodies.

The applicant has now developed a new method of treatment, which is particularly effective in significantly delaying or even inhibiting the immune system reaction without raising the problem of toxicity.

More precisely, the present invention derives from the demonstration of a particularly important synergistic effect related to the combined use of a recombinant adenovirus in which the expression of a gene of therapeutic interest is coupled with that of an immunoprotective gene, as described above, and at least an immunosuppressive agent.

A first objective of this invention, therefore, pertains to a medicinal association of at least an immunosuppressive agent and at least a recombinant adenovirus the genome of which includes a first

recombinant DNA containing a therapeutic gene and a second recombinant DNA containing an immunoprotective gene, for consecutive, intermittent and/or simultaneous use over time, which is useful for *in vivo* and/or *ex vivo* exogenic transfections.

As indicated above, the invention is based, notably, on the demonstration of a synergistic effect between the activity of the immunosuppressive agent and the effect of the immunoprotective gene expressed on the expression of the therapeutic gene.

This combined use permits a clearly prolonged therapeutic effect and advantageously requires significantly lower doses, notably of the immunosuppressive agent.

As indicated below, the two components of the combined treatment of the present invention can be used consecutively, intermittently and/or simultaneously over time. Preferably, the immunosuppressive agent is injected before and after the injection of the adenovirus. According to this mode of implementation of the present invention, the administration of the immunosuppressive agent can be spaced over time, and more preferably, repeated at regular intervals. In this particular case, the two components are packaged separately. In the case of simultaneous administration, they can be mixed extemporaneously before being administered together, or on the other hand, administered simultaneously, but in a separate manner. In particular, the routes of administration of the two agents can be different.

According to the present invention, it is possible to use as the immunosuppressive agent any compound that is capable of partially or completely inhibiting at least one immune signaling pathway. In particular, it can be selected from cyclosporin, FK506, azathioprine, corticosteroids and any monoclonal or polyclonal antibody. These are preferably antibodies capable of inactivating the immune molecules or of causing the destruction of the immune cells carrying these molecules. Notably, it is possible to use as antibodies, anti-CD4, -CD3, CD2, -CD8, -CD28, -B7, -ICAM-1, -LFA-1. It is also possible to use hybrid molecules such as CTLA4Ig, a fusion protein between the CTLA-4 molecule (a CD28 homolog) and an immunoglobulin. The GlFc site of this molecule, by binding to the B7 molecule, is known to be capable of inhibiting the activation of T cells (D.J. Lenschow; Science, 257, 789, 1992). It is clear that the scope of the present invention is in no way limited to the immunosuppressive agents enumerated above. These immunosuppressive agents can be used in isolated form or combined.

With regard to the recombinant DNAs present in the genome of the adenovirus used according to the present invention, they are DNA fragments that contain the gene in question (therapeutic or immunoprotective) and possibly signals that permit their expression, constructed *in vitro* and then inserted into the genome of the adenovirus. The recombinant DNA used as part of the present invention can be complementary DNA (cDNA), genomic DNA (gDNA) or hybrid constructions consisting, for example, of a cDNA into which one or more introns would be inserted. It may also involve synthetic or semisynthetic sequences. These DNAs can be of human, animal, plant, bacterial, or viral, etc. origin. The use of cDNA or gDNA is particularly advantageous.

As the therapeutic gene that can be used for the construction of the vectors of the present invention, it is possible to cite any gene encoding for a product that has a therapeutic effect. The coded product can be a protein, a peptide, an RNA, etc.

If it is a protein product, it can be homologous to the target cell (that is, a product that is normally expressed in the target cell when the latter presents no pathology). In this case, the expression of a protein, for example, makes it possible to overcome insufficient expression in the cell or the expression of an inactive or weakly active protein because of a modification or even to over-express said protein. The therapeutic gene can also encode for a mutant of a cellular protein, which has increased stability, modified activity, etc. The protein product can also be heterologous to the target cell. In this case, a protein expressed, for example, to complete or to provide activity that is lacking in the cell, permitting it to combat a pathology or to stimulate an immune response.

Among the therapeutic protein products in the terms of the present invention, it is possible to cite, more particularly, enzymes, blood derivatives, hormones, interleukins, interferons, TNF [tumor necrosing factor], etc. (Fr 92 03120), growth factors, neurotransmitters or their precursors or synthesis enzymes, trophic factors: BDNF, CNTF, NGF [nerve growth factor], IGF [insulin-like growth factor], GMF, aFGF [fibroblast growth factor], bFGF, NT3, NT5, HARP/pleiotropin, etc.; apolipoproteins: ApoAI, ApoAIV, ApoE, etc. (Fr 93 05125), dystrophin or a mini-dystrophin (Fr 91 11947), the CFTR [Cystic Fibrosis Transmembrane Regulator] protein which is associated with cystic fibrosis, tumor suppressive genes:

p53, Rb, Rap1A, DCC, k-rev, etc. (Fr 93 04745), genes encoding for factors involved in coagulation: Factors VII, VIII, IX, genes intervening in DNA repair, etc.

As indicated above, the therapeutic gene can also be an antisense gene or sequence, and its expression in the target cell controls the expression of genes or the transcription of cellular mRNA. Such sequences can, for example, be transcribed in the target cell by complementary RNA of cellular mRNA, and thus block their translation to protein, according to the technique described in EP patent 140 308. The antisense [sequences] also include sequences coding for ribozymes, which are capable of selectively destroying the target RNAs (EP 321 201).

Therapeutic genes can be of human, animal, plant, bacterial or viral, etc. origin. They can be obtained by any technique know to those skilled in the art, and notably, by screening libraries, by chemical synthesis, or even by combined methods including the chemical or enzymatic modification of sequences obtained by library screening .

Different types of immunoprotective genes can be used within the scope of the present invention. As explained previously, this is a gene whose product acts on the activity of the major histocompatibility complex (MHC) or on the activity of cytokines. It is preferably a gene whose product at least partially inhibits the expression of the MHC proteins or the introduction of antigens. Certain genes contained in the E3 region of the adenovirus, the ICP47 gene of the herpes virus, or the UL18 gene of the cytomegalovirus can be cited as preferred examples.

The E3 region of the adenovirus genome contains different reading phases which, by alternative splicing give rise to different proteins. Among the latter, protein Gp19k (or E3-19k) is a glycosylated transmembrane protein located in the membrane of the endoplasmic reticulum (ER). This protein includes a luminal field binding the MHC-I molecules and a C-terminal cytoplasmic end capable of binding the microtubules (or tubulin) which act to anchor protein gp19k in the ER membrane. Gp19k is thus capable of preventing the expression of MHC-I molecules on the surface of the cells by interaction and sequestration at the level of the ER. However, in the absence of viral replication, protein gp12k is weakly expressed by the adenovirus. Moreover, the expression of gp19k is also dependent on splicing. The introduction into the vectors of the invention of a recombinant DNA containing a sequence

(preferably cDNA) encoding for gp19k makes it possible to control and to optimize the expression of said protein. In particular, the use of constitutive promoters and the suppression of other reading phases make it possible to increase greatly the expression of this protein and to free it from dependence on viral replication and the presence of inducing elements. This is particularly advantageous in considerably reducing lysis of infected cells by CTLs [cytotoxic T-lymphocytes], thus increasing and prolonging the *in vivo* production of the therapeutic gene.

Other proteins encoded by the E3 region of the genome of the adenovirus such as proteins 10,4k and 14,5k represent certain advantageous properties in view of their incorporation in the vectors of the invention.

The ICP47 gene of the herpes simplex virus constitutes another immunoprotective gene that is particularly advantageous in terms of the present invention. Cells infected by the herpes simplex virus are resistant to the lysis induced by the CTLs. It has been demonstrated that this resistance might be conferred by the ICP47 gene, which is capable of reducing the expression of MHC –I molecules on the surface of the cells. The incorporation of the ICP47 gene in a recombinant DNA according to the invention also permits the recombinant viruses of the invention to escape the immune system.

The UL18 gene of the cytomegalovirus is another preferred example of the immunoprotective gene according to the invention. The product of the UL18 gene is capable of binding ß2-microglobulin (Browne et al. Nature 347 (1990) 7707). The ß2-microglobulin is one of the chains of MHC-I molecules. The incorporation of the UL18 gene into a recombinant DNA according to the invention thus makes it possible to reduce the number of functional ß2-microglobulin molecules in the cells infected by the viruses of the invention, and thus to diminish the capacities of these cells to produce complete and functional MHC-I molecules. This type of construction, therefore, protects the infected cells from lysis by CTLs.

As indicated above, the immunoprotective gene used as part of the present invention is, in another preferred mode of embodiment, a gene whose product inhibits the activity of the signaling pathways of cytokines. Cytokines are a family of secreted proteins which act as signaling molecules for the immune

system. They can attract immune cells, activate them, and induce their proliferation, and they can even act directly on the infected cells to kill them.

Among the genes whose product affects the activity or signaling pathways of cytokines, genes that are involved in the synthesis of cytokines, or those whose product is capable of sequestering cytokines, of antagonizing their activity or interfering with intercellular signaling pathways can be cited. As preferred examples, in particular the BCRF1 gene of the Epstein Barr virus, the crmA and crmB genes of the cowpox virus, the B15R and B18R genes of the vaccinia virus, the US28 gene of the cytomegalovirus, and the E3-14,7, E3-10,4 and E3-14,5 genes of the adenovirus can be cited.

The B15R gene of the vaccinia virus encodes for a soluble protein that is capable of binding interleukin-1ß (the secreted form of interleukin-1), and thus preventing this cytokine from binding to its cellular receptors. Interleukin-1 is actually one of the first cytokines produced in response to antigenic aggression, and it plays a very important role in the signaling of the immune system at the beginning of the infection. The possibility of incorporating the B15R gene into a vector according to the invention advantageously makes it possible to reduce the activity if IL-1ß, notably on the activation of the immune cells, and therefore, to locally protect the cells infected by the viruses of the invention against an important immune response. Genes that are homologous to the B15R gene can also be used, such as the ---gene of the cowpox virus.

In the same manner, the B18R gene of the vaccinia virus encodes for a protein that is homologous to the interleukin-6 receptor. This gene, or any functional homolog, can also be used in the vectors of the invention to inhibit interleukin-6 from binding to its cellular receptor, and thus to reduce the immune response locally.

Again, in the same manner, the crmB gene of the cowpox virus can advantageously be used. This gene actually encodes for a secreted protein that is capable of binding TNF and of entering into competition with the TNF receptors on the cell surface. Therefore, in the viruses of the invention, this gene makes it possible to reduce locally the concentration of active TNF that can destroy the infected cells. Other genes encoding for proteins that are capable of binding TNF and at least partially inhibiting it from binding to its receptors can also be used.

The crmA gene of the cowpox virus encodes for a protein that has activity that inhibits serine- type proteases, and which is capable of inhibiting interleukin-1ß synthesis. This gene can be used, therefore, to reduce the local concentration of interleukin-1, and thus to reduce the development of the immune and inflammatory response.

The BCRF1 gene of the Epstein Barr virus encodes for an analog of interleukin 10. The product of this gene is a cytokine that is capable of reducing the immune response and changing its specificity, while inducing the proliferation of B lymphocytes.

The US28 gene of the cytomegalovirus encodes for a protein that is homologous to the 1α-macrophage inflammatory protein (MIP-1α). Therefore, this protein is capable of acting as a competitor of MIP receptors, and thus of inhibiting their activity locally.

The product of genes E3-14,7, E3-10,4 and E3-14,5 of the adenovirus is capable of blocking the transmission of the intercellular signal mediated by certain cytokines. When the cytokines bind to their receptor on the surface of an infected cell, a signal is transmitted to the nucleus to induce cell death or to stop protein synthesis. In particular, this is the case with tumor necrosis factor (TNF). The incorporation of genes E3-14,7, E3-10,4 and/or E3-14,5 into a recombinant DNA according to the invention in view of their constitutive or regulated expression makes it possible to block the intercellular signaling induced by the TNF, and thus to protect the cells infected by the recombinant viruses of the invention from the toxic effects of this cytokine.

Local and transient inhibition can be particularly advantageous. It can be obtained notably by the choice of special expression signals (cytokine-dependent promoters, for example) as indicated below.

It is understood that other homologous genes or those having similar functional properties can be used for the construction of the vectors of the invention. These different genes can be obtained by any technique known to those skilled in the art, and notably by library screening, by chemical synthesis, or even by combined methods that include chemical or enzymatic modification of the sequences obtained by library screening. Moreover, these different genes can be used alone or in combination(s).

The insertion of the genes in question in the form of recombinant DNA according to the invention offers greater flexibility in the construction of adenoviruses, and permits better control of the expression of said genes.

Thus, the recombinant DNAs (and therefore, the genes of interest) incorporated into adenoviral vectors according to the present invention can be organized in different ways.

First of all, they can be inserted into the same site of the adenovirus genome, or different, selected sites. In particular, recombinant DNA can be inserted at least partly in the E1, E3 and/or E4 regions of the adenovirus genome, replacing or supplementing viral sequences.

Preferably, recombinant DNA is inserted, at least partly, in the E1, E3 or E4 regions of the adenovirus genome. When it is inserted at two different sites, within the scope of the invention, it is preferred to use regions E1 and E3 or E1 and E4. The examples actually show that this organization permits a high expression of the two genes, without interference between the two. Advantageously, the recombinant DNAs are inserted by replacing viral sequences.

Each recombinant DNA can then include an identical or different transcriptional promoter. This configuration makes it possible to obtain higher levels of expression and to offer better control of the expression of the genes. In this case, the two genes can be inserted in the same orientation or in opposite orientations.

They can also constitute an unique transcriptional entity. In this configuration, two recombinant DNAs are contiguous and positioned such that the two genes are under the control of a single promoter, and give rise to a unique premessenger RNA. This arrangement is advantageous since it uses a single transcriptional promoter.

Finally, the use of recombinant DNA according to the invention makes it possible to use different types of transcriptional promoters, and notably, promoters that are strong or weak, regulated or constitutive, tissue-specific or ubiquitous, etc.

The choice of expression signals and of the respective position of the recombinant DNA is particularly important in obtaining a high expression of the therapeutic gene and an important immunoprotective effect.

A particularly preferred embodiment of the present invention makes use of a defective adenovirus comprising a first recombinant DNA containing a therapeutic gene and a second recombinant DNA containing an immunoprotective gene, in which the two recombinant DNAs are inserted in the E1 region.

A particularly preferred embodiment of the present invention uses a defective adenovirus comprising a first recombinant DNA containing a therapeutic gene, inserted in the E1 region, and a second recombinant DNA containing an immunoprotective gene inserted in the E3 region.

As indicated above, the adenoviruses of the present invention are defective, that is, they are incapable of replicating autonomously in the target cell. Generally, therefore, the genome of defective adenoviruses according to the present invention lacks at least the sequences necessary for the replication of said virus in the infected cell. These regions can either be eliminated (completely or partly), or rendered non-functional, or substituted by other sequences, and notably by therapeutic genes. The defective character of the adenoviruses of the invention is an important element since it ensures non-dissemination of the vectors of the invention after administration.

In a preferred method of embodiment, the adenoviruses of the invention comprise ITR sequences and a sequence that permits encapsidation, and they possess a deletion of all or part of the E1 gene.

Repeated inverse sequences (ITR) are the source of adenovirus replication. They are located at the 3' and 5' ends of the viral genome (cf Figure 1), where they can easily be isolated according to classic molecular biology techniques known to those skilled in the art. The nucleotide sequence of the ITR sequences of human adenoviruses (in particular, serotypes Ad2 and Ad5) is described in the literature, as well as that of canine adenoviruses (notably, CAV1 and CAV2). With regard to adenovirus Ad5, for example, the left ITR sequence corresponds to the region that includes nucleotides 1 to 103 of the genome.

The encapsidation sequence (also called the Psi sequence) is necessary for the encapsidation of viral DNA. This region must be present to permit the preparation of the defective recombinant adenoviruses according to the invention. The encapsidation sequence is located in the genome of the adenoviruses, between the left ITR (5') and the E1 gene (Cf Figure 1). It can be isolated or synthesized artificially by classic molecular biology techniques. The nucleotide sequence of the encapsidation sequence of human adenoviruses (in particular, serotypes Ad2 and Ad5) is described in the literature, as well as that of canine adenoviruses (notably CAV1 and CAV2). With regard to the Ad5 adenovirus, for example, the encapsidation sequence corresponds to the region comprising nucleotides 194 to 358 of the genome.

More preferably, the adenoviruses of the invention comprise the ITR sequences and a sequence that permits encapsidation and they have a deletion of all or part of the E1 and E4 genes.

In a particularly preferable embodiment according to the invention, all or part of the E1, E3 and E4 genes is deleted from the genome of the adenovirus, and even more preferably, all or part of genes E1, E3, L5 and E4.

The adenoviruses of the invention can be prepared from adenoviruses of different origins. In fact, there are different types of adenovirus serotypes, in which the structure and properties vary somewhat, but which have a comparable genetic organization. Thus, the information described in the present application can easily be reproduced by those skilled in the art for any type of adenovirus.

More particularly, the adenoviruses of the invention can be of human, animal or combined (human and animal) origin.

With regard to adenoviruses of human origin, it is preferable to use those classified in group C. More preferably, among the different human adenovirus serotypes, within the context of the present invention, it is preferable to use type 2 or 5 (Ad2 or Ad5) adenoviruses.

As indicated above, the adenoviruses of the invention can also be of animal origin, or can include sequences from adenoviruses of animal origin. In fact, the applicant has demonstrated that adenoviruses of animal origin are extremely efficacious in infecting human cells, and that they are incapable of propagating in the human cells in which they have been tested (Cf Application FR 93 05954). The

applicant has also demonstrated that adenoviruses of animal origin are in no way trans-complemented by adenoviruses of human origin, which eliminates any risk of recombination and propagation *in vivo*, in the presence of a human adenovirus, which might lead to the formation of an infectious particle. The use of adenoviruses or of adenovirus regions of animal origin is, therefore, particularly advantageous since the risks inherent in the use of viruses as vectors in gene therapy are even lower.

Adenoviruses of animal origin that can be used within the context of the present invention can be of canine, bovine, murine (example: Mav1, Beard et al., Virology 75 (1990) 81), ovine, porcine, avian or even simian (example: SAV)origin. More particularly, among the avian adenoviruses, ATCC accessible serotypes 1 to 10 , such as for example, Phelps (ATCC VR-432), Fontes (ATCC VR-280), P7-A (ATCC VR-827), IBH-2A (ATCC VR-828), J2-A (ATCC VR-829), T8-A (ATCC VR-830), K-11 (ATCC VR-921), and also ATCC referenced strains VR-831 to 835 can be cited. Among the bovine adenoviruses, different known serotypes can be used, and notably those accessible at the ATCC (types 1-8) under references ATCC VR-313, 314, 639-642, 768 and 769. The following murine adenoviruses can also be cited: FL (ATCC VR-550) and E20308 (ATCC VR-528), ovine adenovirus type 5 (ATCC VR-1343), or type 6 (ATCC VR-1340); porcine adenovirus 5359) [sic], or simian adenoviruses such as, in particular, ATCC adenoviruses referenced as numbers VR-591-594, 941-943, 195-203, etc.

Preferably, among the different adenoviruses of animal origin, within the context of the invention, adenoviruses or adenovirus regions of canine origin are used, and particularly all strains of CAV2 adenoviruses [Manhattan strain or A26/61 (ATCC VR-800), for example]. Canine adenoviruses have been the subject of several structural studies. Thus, complete restriction maps of the CAV1 and CAV2 adenoviruses have been described in the prior art (Spibey et al., J. Gen. Virol. 70 (1989) 165), and E1a, E3 genes, as well as ITR sequences have been cloned and sequenced (see, in particular, Spibey et al., Virus Res. 14 (1989) 241; Linné, Virus Res. 23 (1992) 199, WO 91/11525).

The defective recombinant adenoviruses according to the invention can be prepared in different ways.

A first method consists of transfecting the DNA from the defective recombinant virus prepared *in vitro* (either by splicing or in plasmid form) into a competent cell line, that is, one that has all of the functions necessary for complementation of the defective virus in the *trans* configuration. These functions are preferably integrated into the cell's genome, which makes it possible to avoid the risks of recombination, and provides the cell line with increased stability.

A second approach consists of cotransfecting into an appropriate cell line the DNA of the defective recombinant virus prepared *in vitro* (either by splicing, or in plasmid form) and the DNA of a helper virus. According to this method, it is not necessary to have a competent cell line capable of complementing all of the defective functions of the recombinant virus. Some of these functions are, in fact, complemented by the helper virus. This helper virus must itself be defective and the cell line has the functions that are necessary for its complementation in the *trans* configuration. Among the cell lines that can be used within the scope of this second approach, the human embryo kidney line 293, KB [human oral epidermoid carcinoma] cells, HeLa cells, MDCK [Madin-Darby canine kidney], GHK, etc. can be cited (Cf examples).

Next, the vectors which are multiplied are recovered, purified and amplified according to classic molecular biology techniques.

According to one embodiment variation, it is possible to prepare *in vitro*, either by splicing or in plasmid form, the DNA of the defective recombinant virus carrying the appropriate deletions and the two recombinant DNAs. As indicated above, the vectors of the invention advantageously possess a deletion of all or part of certain viral genes, notably genes E1, E3, E4 and/or L5. This deletion may correspond to any type of suppression affecting the gene in question. It may, in particular, involve suppression of all or part of the encoding region of said gene, and/or all or part of the transcription promoting region of said gene. Suppression generally takes place on the DNA of the defective recombinant virus, for example, by digestion by the appropriate restriction enzymes, then splicing, according to molecular biology techniques, as illustrated in the examples. The recombinant DNA can then be inserted into that DNA by enzyme cleavage then splicing, in the selected regions and in the chosen orientation.

DNA obtained in this manner, which therefore carries the appropriate deletions and the two recombinant DNAs, makes possible direct generation of the defective recombinant adenovirus carrying said deletions and recombinant DNA. This first variation is particularly well-suited to the embodiment

of the recombinant adenoviruses in which the genes are arranged in the form of a unique transcriptional unit, or under the control of separate promoters, but inserted into a single site on the genome.

It is also possible to prepare the recombinant virus in two steps, permitting the successive introduction of two recombinant DNAs. Thus, the DNA from a first recombinant virus carrying the appropriate deletions (or some of said deletions) and one of the recombinant DNAs is constructed, by splicing or in plasmid form. This DNA is then used to generate a first recombinant virus carrying said deletions and a recombinant DNA. The DNA from this first virus is then isolated and cotransfected with a second plasmid or the DNA from a second defective recombinant virus carrying the second recombinant DNA, the appropriate deletions (the part that is not present on the first virus), and a region that permits homologous recombination. This second step thus generates the defective recombinant virus carrying the two recombinants DNAs. This preparation variant is particularly appropriate for the preparation of recombinant viruses carrying two recombinant DNAs inserted in two different regions of the adenovirus genome.

The two agents according to the invention, namely the immunosuppressive agent and the recombinant adenovirus can be formulated with a view to topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular, transdermal, etc. administration.

Preferably, the respective pharmaceutical formulation or formulations contain vehicles that are pharmaceutically acceptable for an injectable formulation. In particular, they can be sterile, isotonic saline solutions (monosodium phosphate, disodium phosphate, sodium, potassium, calcium or magnesium, etc. chloride, or mixtures of these salts), or dry compositions, notably freeze dried, which by adding sterilized water or physiological serum, according to the case, permits the constitution of injectable solutions.

The doses of immunosuppressive agent and adenoviruses used for the injection can be adjusted as a function of different parameters, and notably, as a function of the method of administration used, the pathology in question, the gene to be expressed, or even the desired duration of the treatment.

In general, recombinant adenoviruses according to the invention are formulated and administered in the form of doses of between 10^4 and 10^{14} pfu/ml, and preferably 10^6 to 10^{10} pfu/ml. The term pfu (plaque-forming unit) represents the infectious power of a particular solution, and it is determined by infection of an appropriate cell culture, and measurement, generally after 5 days, of the number of plaques of infected cells. Methods of determining the pfu titer of a viral solution are well documented in the literature. More particularly, with regard to immunosuppressive agents, the doses and injection methods for these agents vary according to their nature. The adjustment of these parameters is within the competence of those skilled in the art.

The medicinal association according to the invention can be used for the treatment or prevention of numerous pathologies. Depending on the therapeutic gene that is inserted into its adenovirus, it can be used, notably, for the treatment or prevention of genetic diseases ([muscular] dystrophy, cystic fibrosis, etc., neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, ALS, etc.), hyperproliferative diseases (cancers, restenosis, etc.), diseases related to coagulation disorders or to dyslipoproteinemias, diseases related to viral infections (hepatitis, AIDS, etc.), etc.

The present invention also applies to any method of therapeutic treatment that implements the claimed medicinal association.

The present invention will be more fully described with the help of the following examples, which should be regarded as illustrative and not limiting.

Figure 1: Genetic organization of the Ad5 adenovirus. The complete Ad5 sequence is available on the data base and permits those skilled in the art to select or to create any restriction site, and thus to isolate any region of the genome.

Figure 2: Restriction map of the Manhattan strain CAV2 adenovirus (according to the aforementioned Spibey et al.).

Figure 3: Construction of the pAD5-gp19k-ßgal vector.

Figure 4: Construction of the Ad-gp19k-ßgal,ΔE1, ,ΔE3 adenovirus.

General Molecular Biology Techniques

The classic methods used in molecular biology, such as preparative extractions of plasmid DNA, centrifugation of plasmid DNA in a cesium chloride gradient, electrophoresis on agarose or acrylamide gels, purification of DNA fragments by electroelution, extraction of proteins with phenol or phenol-chloroform, DNA precipitation in saline medium by ethanol or isopropanol, transformation in *Escherichia coli*, etc. are well known to those skilled in the art and are fully described in the literature [Maniatis T. et al., "Molecular Cloning, a Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982; Ausubel F.M. et al. (eds), "Current Protocols in Molecular Biology", John Wiley & Sons, New York, 1987].

Type pBR322, pUC plasmids and series M13 phages can be obtained from commercial sources (Bethesda Research Laboratories).

For splicing, the DNA fragments can be separated according to their size by agarose or acrylamide gel electrophoresis, extracted with phenol or by a phenol-chloroform mixture, precipitated with ethanol, then incubated in the presence of T phage DNA ligase (Biolabs) according to the supplier's recommendations.

Filling of the proeminent 5' ends can be effected by the Klenow fragment of DNA Polymerase I of *E. coli* (Biolabs) used according to the manufacturer's recommendations. The destruction of the proeminent 3' ends takes place in the presence of T4 phage DNA polymerase (Biolabs) used according to the manufacturer's recommendations. The destruction of the proeminent 5' ends is carried out by a treatment controlled by the S1 nuclease.

Mutagenesis directed *in vitro* by synthetic oligodeoxynucleotides can be carried out according to the method developed by Taylor et al. [Nucleic Acids Res. 13 (1985) 8749-8764] using the kit distributed by Amersham.

Enzyme amplification of DNA fragments by the so-called PCR method [Polymerase-catalyzed Chain Reaction, Saiki R.K. et al., Science 230 (1985) 1350-1354; Mullis K.B. and Faloona F.A., Meth. Enzym. 155 (1987) 335-350] can be carried out using a "DNA thermal cycler" (Perkin Elmer Cetus) according to the manufacturer's specifications.

Verification of nucleotide sequences can be carried out by the method developed by Sanger et al. [Proc. Natl. Acad. Sci. USA, 74 (1977) 5463-5467) using the kit distributed by Amersham.

Cell Lines Used

In the following examples, the following cells lines have been used or could be used:

- Human embryo kidney line 293 (Graham et al., J. Gen. Virol. 36 (1977) 59). This line contains, notably, integrated into its genome, the left section of the genome of the human adenovirus Ad5 (12%).

- KB Human cell line: Obtained from human epidermal carcinoma, this line is accessible to the ATCC (ref. CCL17) as well as the conditions for its culture.

- HeLa human cell line: Obtained from human epithelium carcinoma, this line is accessible to the ATCC (ref. CCL2) as well as the conditions for its culture.

- MDCK canine cell line: The conditions for culturing these MDCK cells have been described, notably by Macatney et al., Science 44 (1988) 9.

- gm DBP6 cell line (Brough et al., Virology 190 (1992) 624). This line consists of HeLa cells carrying the adenovirus E2 gene under the control of LTR [Long Term Repeat] and MMTV [Mouse Mammary Tumor Virus].

EXAMPLES

Example 1. Construction of defective recombinant adenoviruses comprising a therapeutic gene (the LacZ gene of *E. coli*) under the control of the LTR promotor of the RSV and the gp19k gene under the control of the RSV LTR promoter, both inserted in the E1 region.

These adenoviruses have been constructed by homologous recombination between a plasmid carrying the left section of the Ad5 adenovirus, the two recombinant DNAs and a region of the Ad5 adenovirus (corresponding to protein IX) and the DNA of a defective adenovirus having different deletions.

1. Construction of the pAD5-gp19k-ßgal vector (Figure 3)

1.1 Construction of the pGEM-gp19k plasmid

The pAD5-gp19k-ßgal plasmid contains a cDNA sequence encoding for the adenovirus gp19k protein. This plasmid was constructed as follows. The XbaI fragment of the wild-type adenovirus Ad5

genome containing the E3 region was isolated and cloned at the corresponding site of the pGEM plasmid (Promega) to generate the pGEM-E3 plasmid. The HinfI fragment containing the gp19k encoding sequence (nucleotides 28628 to 29634 of the wild-type Ad5 adenovirus) was then isolated from the pGEM-E3 plasmid. The ends of this fragment were rendered free by the action of the Klenow fragment of DNA polymerase I of *E. coli* (Cf General Molecular Biology Techniques), then the fragment obtained was cloned at the SmaI site of the pGEMzf+ plasmid (Promega).

The plasma obtained was designated pGEM-gp19k (Figure 3).

1.2. Construction of the pAD5-gp19k-ßgal vector

This example describes the construction of a plasmid containing one of the two recombinant DNAs comprising their own promoter, the left section of the adenovirus genome and a supplementary part (protein pIX) permitting homologous recombination. This vector was constructed from the pAd.RSVßGal plasmid as follows.

The pAd.RSVßGal plasmid contains, in the 5'->3' orientation,

- the PvuII fragment corresponding to the left end of the Ad5 adenovirus comprising: the ITR sequence, the replication source, the encapsidation signals and the E1A amplifier;

- the gene encoding for ß-galactosidase under the control of the RSV (Rous sarcoma virus) promoter,

- a second fragment of the Ad5 adenovirus genome, which permits homologous recombination between the pAd.RSVßGal plasmid and adenovirus d1324. The pAD.RSVßGal was described by Stratford-Perricaudet et al. (J. Clin. Invest. 90 (1992) 626).

Plasmid pAd.RSVßGal was first cut by the EagI and ClaI enzymes. This generated a first fragment carrying notably the left part of the Ad5 adenovirus and the LTR promoter of the RSV. In parallel, plasmid pAd.RSVßGal was also cut by the EagI and XabI enzymes. This generated a second type of fragment carrying notably the LTR promoter of the RSV, the LacZ gene, and a fragment of the Ad5 adenovirus, which permits homologous recombination. The ClaI-EagI and EagI-XbaI were then spliced

in the presence of the XbaI-ClaI fragment of the pGEM-gp19k plasmid (example 1.1) carrying the coding sequence of gp19k (Cf Figure 3). The vector obtained, which was called pAD5-gp19k-ßgal, contained, therefore,

- the PvuII fragment corresponding to the left end of the Ad5 adenovirus comprising: the ITR sequence, the replication source, the encapsidation signals and the E1A amplifier;

- the sequence encoding for gp19k under the control of the RSV promoter (of the Rous sarcoma virus);

- the gene encoding for ß-galactosidase under the control of the RSV promoter (of the Rous sarcoma virus), and

- a second fragment of the Ad5 adenovirus genome, which permits homologous recombination.

2. Construction of recombinant adenoviruses

2.1. Construction of a recombinant adenovirus deleted in the E1 region, having two recombinant DNAs inserted in the same orientation, at the level of the E1 region.

The pAD5-gp19k-ßgal vector was linearized and cotransfected with an adenoviral vector that is deficient in the E1 gene, in helper cells (line 293) changing the functions coded by the E1 regions (E1A and E1B) of the adenovirus to the *trans* configuration.

More specifically, the adenovirus Ad-gp19k-ßgal-ΔE1 is obtained by *in vivo* homologous recombination between the Ad-RSVßgal adenovirus (Cf Stratford-Perricaudet et al. cited above) and the pAD5-gp19k-ßgal vector is obtained according to the following protocol: the pAD5-gp19k-ßgal plasmid, linearized by XmnI, and the Ad-RSVßgal adenovirus, linearized by the ClaI enzyme, are cotransfected into line 293 in the presence of calcium phosphate to permit homologous recombination. The recombinant adenoviruses generated are then selected by plate purification. After isolation, the DNA of the recombinant adenovirus is amplified in cell line 293, which leads to a culture supernatant containing the non-purified defective recombinant adenovirus having a concentration of approximately 10^{10} pfu/ml.

Viral particles are generally purified by centrifugation on a cesium chloride gradient according to known techniques (see, in particular, Graham et al., Virology 52 (1973) 456). The AD-gp19k-ßgal,ΔE1 adenovirus can be stored at –80°C in 20% glycerol.

2.2. Construction of a recombinant adenovirus deleted in the E1 and E3 regions, having two recombinant DNAs inserted in the same orientation, at the level of the E1 region (Figure 4).

The pAD5-gp19k-ßgal vector was linearized and cotransfected with an adenoviral vector that is deficient in the E1 and E3 genes, in helper cells (line 293) and the functions encoded by the E1 regions (E1A and E1B) of the adenovirus were changed to the *trans* configuration.

More specifically, the adenovirus Ad-gp19k-ßgal-ΔE1,ΔE3 was obtained by *in vivo* homologous recombination between the mutant adenovirus Ad-dl1324 (Thimmappaya et al., Cell 31 (1982) 543) and the pAD5-gp19k-ßgal vector, according to the following protocol: the pAD5-gp19k-ßgal plasmid, linearized by XmnI, and the Ad-RSVßgal adenovirus and the Ad-dl1324 adenovirus, linearized by the ClaI enzyme, were cotransfected into line 293 in the presence of calcium phosphate to permit homologous recombination. The recombinant adenoviruses generated were then selected by plate purification. After isolation, the DNA of the recombinant adenovirus was amplified in cell line 293, which produced a culture supernatant containing the non-purified defective recombinant adenovirus having a concentration of approximately 10^{10} pfu/ml.

Viral particles are generally purified by centrifugation on a cesium chloride gradient according to known techniques (see, in particular, Graham et al., Virology 52 (1973) 456). The genome of the recombinant adenovirus is then verified by Southern blot analysis. The AD-gp19k-ßgal,ΔE1, ΔE3 adenovirus can be stored at –80°C in 20% glycerol.

<u>Example 2</u>: Detection of the immunoprotective activity of the medicinal association according to the invention.

Sixty DBA/2 adult female mice were randomly divided into 6 groups of mice treated according to the following respective injection protocols:

- GROUP 1a:

This group received an intraocular injection of 10 μg of anti-CD3 monoclonal antibody on days –2, -1, 1, 2, 3, 4 and 5 with an intravenous injection of 4.10^9 pfu of the Ad-RSVßgal virus on day zero (Cf Stratford-Perricaudet et al. cited above).

- GROUP 1b:

This group received the same treatment as group 1a using as the virus 4.10^9 pfu of the Ad-gp19k-ßgal virus (Figure 4).

- GROUP 2a:

This group received an intrapetitional [sic – possible misprint for "intraperitoneal"] injection of 250 μg of anti-CD4 monoclonal antibody on days –2, -1, 1, 4, 7 with an intravenous injection of 4.10^9 pfu of the Ad-RSV ß gal virus on day zero.

- GROUP 2b:

This group received the same treatment as group 2a using as the virus 4.10^9 pfu of the Ad gp 19k-ßgal virus.

- GROUP 3a:

This group received an intravenous injection of 4.10^9 pfu of Ad-ßgal without the combined administration of an immunosuppressive agent.

- GROUP 3b:

This group received an intravenous injection of 4.10^9 pfu of Ad-gp19k-ßgal without the combined administration of an immunosuppressive agent.

At different times, two animals from each group were sacrificed and their livers and spleens were extracted.

2.1- Determination by FACS [fluorescence activated cell sorter] analysis of the % of splenocytes having the antigen 14 days post-injection.

The spleens were crushed and the splenocytes were extracted. A sample was subjected to FACS analysis to determine the distribution of immune cells. Table I below summarizes the results.

[Page prior to modification]

	Group 3 a Ad-ßgal		Group 3 b Ad-ßgal-gp19K		Group 3 a CD3/Ad-ßgal		Group 2 a CD4/Ad-ßgal	
	% of cells expressing ßgal on the surface of the cell							
CD3	20.4	17.5	20.6	21	5.4	6.1	12	10.3
CD4	13.4	12.6	15.3	16.8	4.4	5.1	2.7	4.1
CD8	5.5	5.5	6.1	6	20.2	23	7.9	6.7

TABLE I

Note the inhibiting effect of the anti-CD3 and anti-CD4 immunosuppressive agents on antibody production.

2.2- Cytotoxicity of splenocytes stimulated 14 days post-injection

The remainder of the cells extracted from the liver were stimulated in the presence of P815-ßgal cells, infected with Ad-ßgal at a MOI [Multiplicity of Infection] of 100 pfu, to amplify any CTL [cytotoxic T-lymphocyte] clone recognizing the ß-galactosidase antigens. After 4 days of stimulation, the cytotoxic activity of the splenocytes was determined, using a ^{51}Cr release cytotoxic test, with labeled P815-ß gal cells used as the target cells. The results are presented in Table II below.

Group 2a (anti-CD4/Ad-ßgal)	Low
Group 2b (anti-CD4/Ad gp19k-ßgal)	Undetectable
Group 3a (Ad-ßgal)	High
Group 3b (Ad-gp19k-ßgal)	Average

TABLE II

Only the group treated according to the invention, namely group 2b, was not associated with any cytotoxic activity.

2.3 Expression of ß-galactosidase activity in the liver after 14 days

The livers were sectioned and stained with X-gal to reveal ß-galactosidase activity and eosin to reveal the section's histology. The results are presented in Table III below.

	Number of cells expressing ßgal	
	14 days	31 days
Group 2a: (anti-CD4/Ad-ßgal)	1	1
Group 2b: (anti-CD4/Ad gp19k-ßgal)	250	50
Group 3a: (Ad-ßgal)	3	0
Group 3b: (Ad-gp19k-ßgal)	25	0

TABLE III

From the results shown above, it is obvious that the injection of anti-CD4 antibodies combined with an injection of Adgp-19k-ßgal induced a clearly prolonged expression of the gene in question. Thirty days after the injections, significant ß-galactosidase activity can be observed in the case of group 2b. This prolongation, which can be interpreted as the result of a tolerance phenomenon induced according to the invention, is clearly superior to that which might be expected from the simple juxtaposition of the respective effects of the anti-CD4 immunosuppressant and the Ad gp198-ßgal recombinant adenovirus.

In addition, no inflammatory reaction was observed during this 30-day period in the case of Group 2b.

CLAIMS

1. Medicinal combination of at least an immunosuppressive agent and at least a recombinant adenovirus the genome of which includes a first recombinant DNA containing a therapeutic gene and a second recombinant DNA containing an immunoprotective gene, for consecutive, intermittent and/or simultaneous use over time, useful for exogenic *in vivo* and/or *ex-vivo* transfections.

2. Medicinal combination according to Claim 1 characterized in that the immunosuppressive agent is preferably selected from cyclosporin, FK506, azathioprine, corticosteroids and monoclonal or polyclonal antibodies.

3. Medicinal combination according to Claim 2 characterized in that it involves antibodies that are capable of inactivating immune molecules and causing the destruction of the immune cells having these molecules.

4. Medicinal combination according to Claim 3 characterized in that the antibody is selected from anti-CD4, -CD2, -CD3, -CD8, -CD28, -B7, -ICAM-1, -LFA-1 and CTLA4Ig.

5. Medicinal combination according to one of the preceding claims characterized in that the therapeutic gene encodes for a therapeutic protein.

6. Medicinal combination according to one of Claims 1 to 4 characterized in that the therapeutic gene encodes for a therapeutic RNA.

7. Medicinal combination according to one of the preceding claims characterized in that the immunoprotective gene is a gene whose product acts on the activity of the major histocompatibility complex (MHC) or on the activity of cytokines.

8. Medicinal combination according to Claim 7 characterized in that the immunoprotective gene is a gene whose product at least partially inhibits the expression of MHC proteins or the introduction of antigens.

9. Medicinal combination according to one of the preceding claims characterized in that the immunoprotective gene is selected from the gp19k gene of the adenovirus, the ICP47 gene of the herpes virus, or the UL18 gene of the cytomegalovirus.

10. Medicinal combination according to one of the preceding claims characterized in that the two recombinant DNAs of the adenovirus genome constitute a unique transcriptional entity.

11. Medicinal combination according to one of the preceding claims characterized in that each one of the two recombinant DNAs has an identical or different transcriptional promoter.

12. Medicinal combination according to Claim 11 characterized in that the two recombinant DNAs are inserted in the same orientation.

13. Medicinal combination according to Claim 11 characterized in that the two recombinant DNAs are inserted in opposite orientations.

14. Medicinal combination according to one of the preceding claims characterized in that the two recombinant DNAs are inserted in the same site of the adenovirus genome, preferably at the level of regions E1, E3 or E4.

15. Medicinal combination according to Claim 14 characterized in that the two recombinant DNAs are inserted at the level of region E1.

16. Medicinal combination according to one of Claims 1 to 13 characterized in that the two recombinant DNAs are inserted into different sites of the adenovirus genome.

17. Medicinal combination according to Claim 16 characterized in that one of the recombinant DNAs is inserted at the level of the E1 region and the other at the level of the E3 or E4 region.

18. Medicinal combination according to one of the preceding claims characterized in that the adenovirus is a defective recombinant adenovirus comprising ITR sequences, a sequence that permits encapsidation, and has a deletion of all or part of the E1 and E4 genes.

19. Medicinal combination according to Claim 18 characterized in that it is an adenovirus comprising ITR sequences, a sequence that permits encapsidation and has a deletion of all or part of the E1, E3 and E4 genes.

20. Medicinal combination according to one of Claims 1 to 19 characterized in that it involves an adenovirus of which the genome is deleted of all or part of genes E1, E3, L5 and E4.

21. Medicinal combination according to one of the preceding claims characterized in that the recombinant adenovirus is of human, animal or mixed origin.

22. Medicinal combination according to Claim 21 characterized in that the recombinant adenoviruses of human origin are selected from those classified in Group C, preferably from type 2 or type 5 recombinant adenoviruses (Ad 2 or Ad 5).

23. Medicinal combination according to Claim 22 characterized in that the adenoviruses of animals origin are selected from adenoviruses of canine, bovine, murine, ovine, porcine, avian and simian origin.

24. Medicinal combination according to one of the preceding claims characterized in that the immunosuppressive agent is injected before and after the injection of the adenovirus.

25. Medicinal combination according to one of the preceding claims characterized in that the immunosuppressive agent and the recombinant adenovirus are injected simultaneously.

[Modified page]

	Group 3 a Ad-ßgal		Group 3 b Ad-ßgal-gp19K		Group 1 a anti CD3/ Ad-ßgal		Group 2 a anti CD4/ Ad-ßgal	
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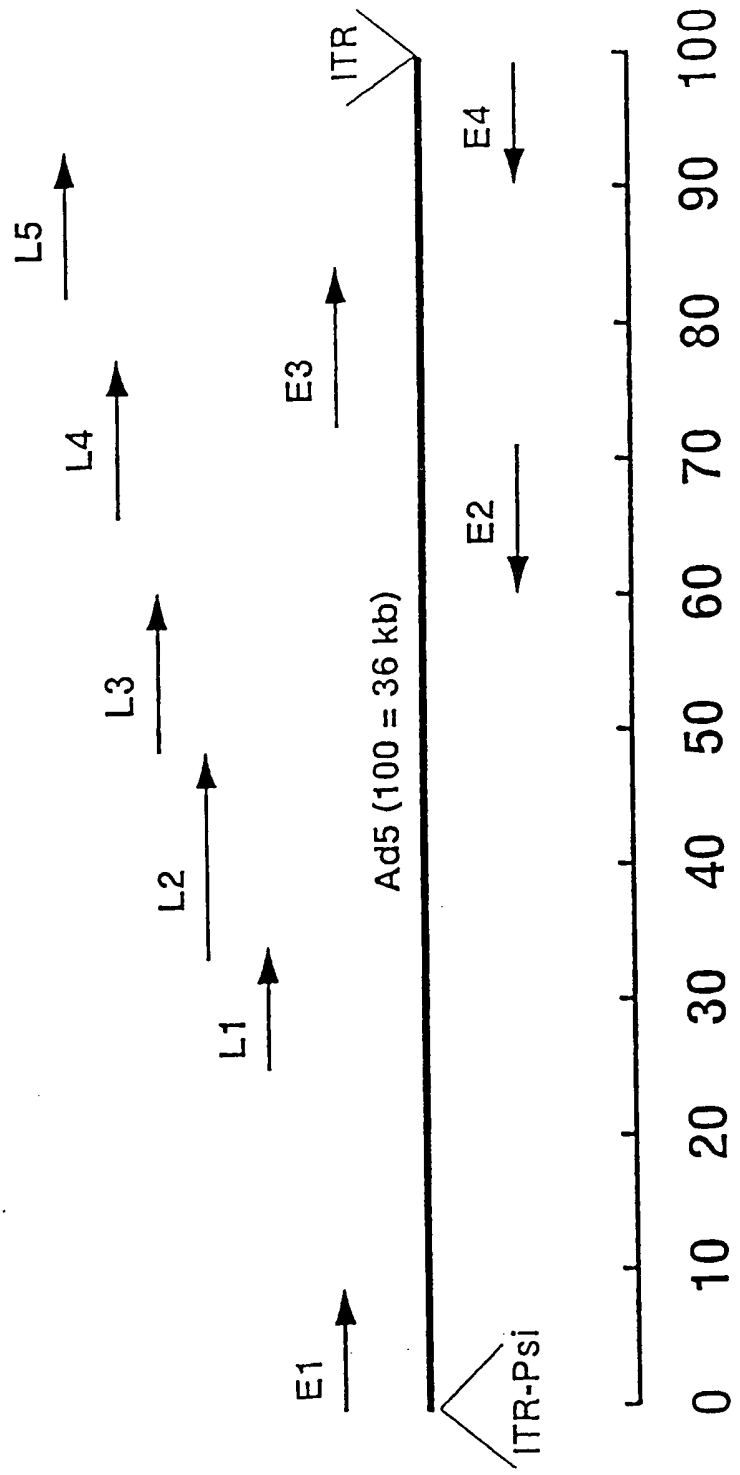
Group 3b (Ad-gp19k-ßgal)	Average

TABLE II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E4
L5
E3
L4
E2
L3
L2
Ad5 (100 = 36 kb)
L1
E1
ITR-Psi
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10
20
30
40
50
60
70
80
90
100

Figure 1

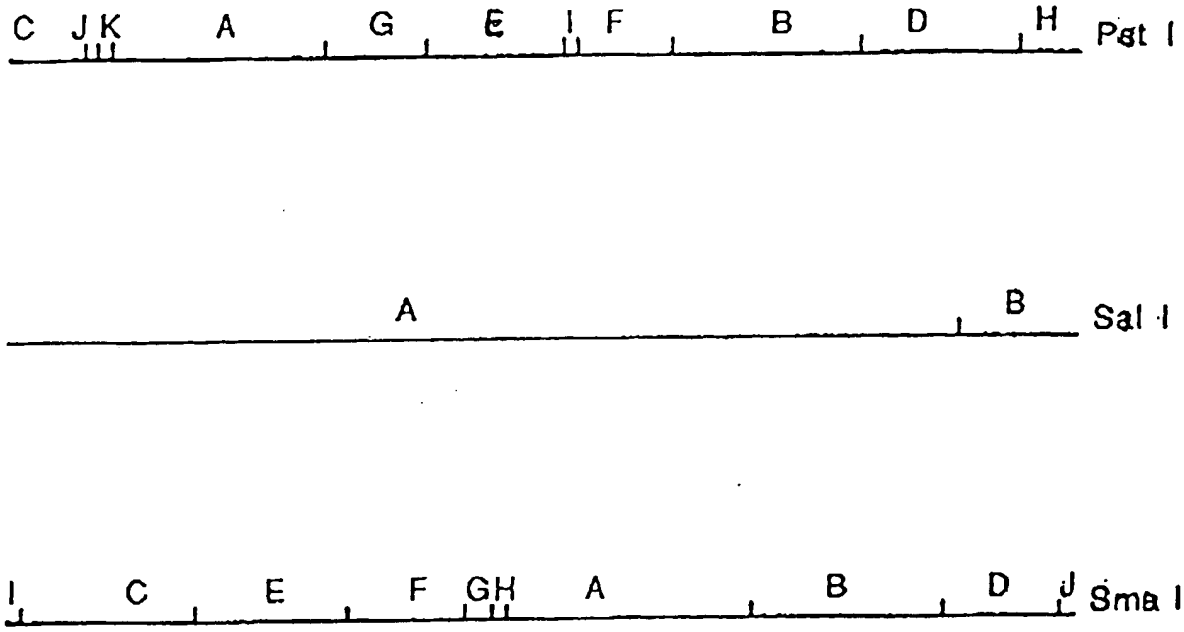
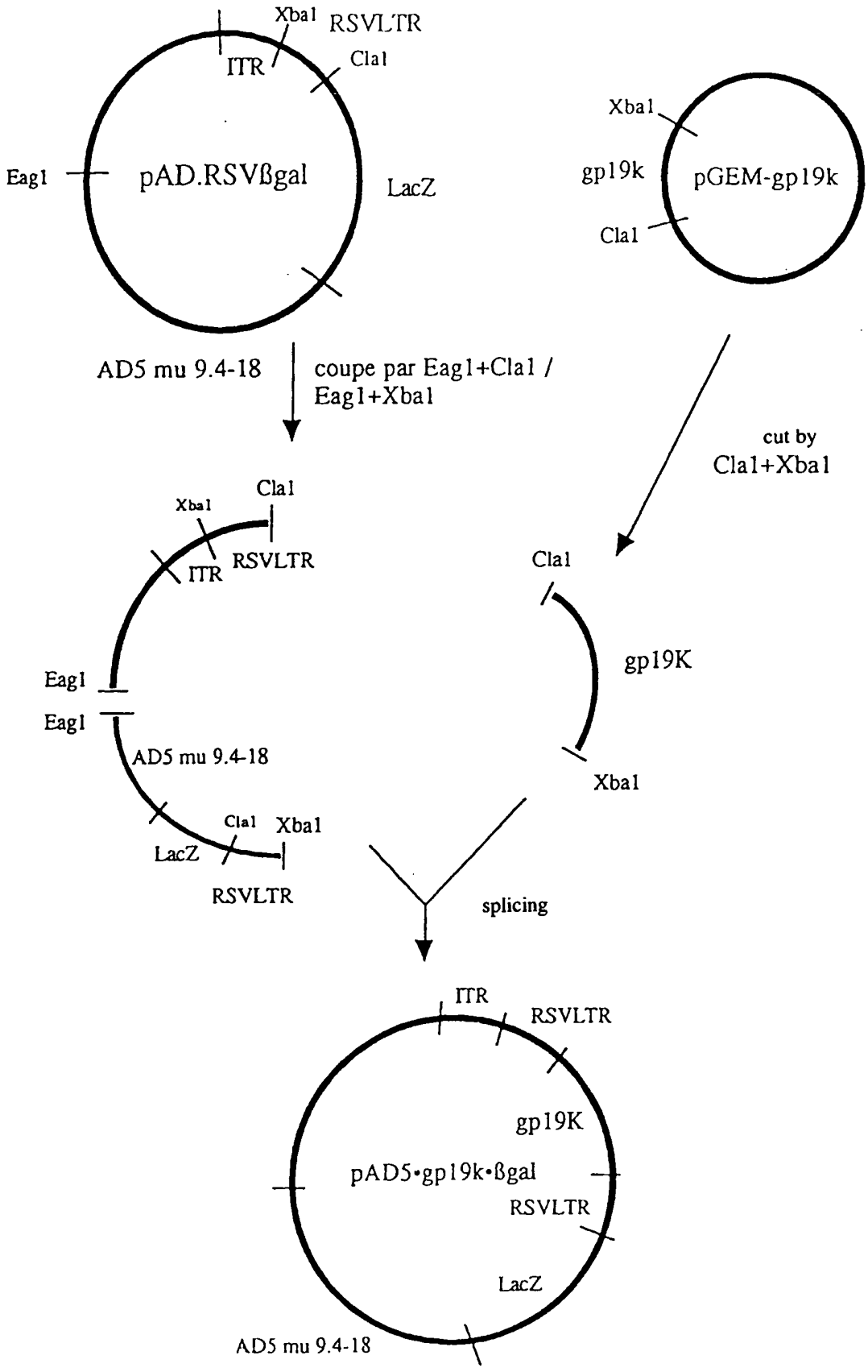
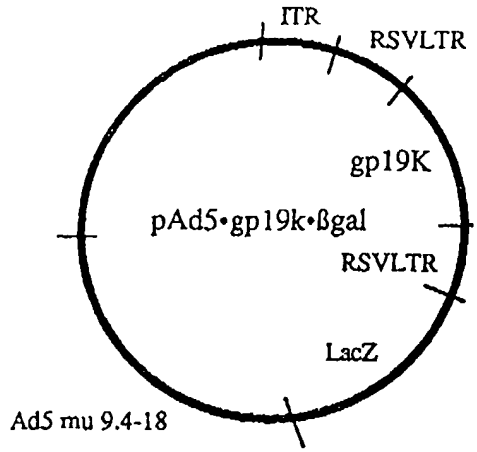
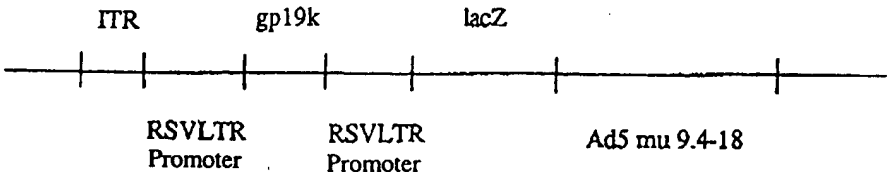


Figure 2


Xba1
RSVLTR
ITR
Cla1
Eag1
pAD.RSVßgal
LacZ
Xba1
gp19k
pGEM-gp19k
Cla1
AD5 mu 9.4-18
coupe par Eag1+Cla1 / Eag1+Xba1
cut by Cla1+Xba1
Cla1
Xba1
ITR
RSVLTR
Eag1
Eag1
AD5 mu 9.4-18
Cla1
Xba1
LacZ
RSVLTR
Cla1
gp19K
Xba1
splicing
ITR
RSVLTR
gp19K
pAD5•gp19k•ßgal
RSVLTR
LacZ
AD5 mu 9.4-18



Linearization



Recombination

D1324 genome

Ad-gp19K-lacZ